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Detection of Purple Sulfur Bacteria in Purple and Non-purple Dairy Wastewaters

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Abstract

The presence of purple bacteria in manure storage lagoons is often associated with reduced odors. In this study, our objectives were to determine the occurrence of purple sulfur bacteria (PSB) in seven dairy wastewater lagoons and to identify possible linkages between wastewater properties and purple blooms. Community DNA was extracted from composited wastewater samples, and a conservative 16S rRNA gene sequence within *Chromatiaceae* and *pufM* genes found in both purple sulfur and nonsulfur bacteria was amplified. Analysis of the genes indicated that all of the lagoons contained sequences that were 92 to 97% similar with *Thiocapsa roseopersicina*. Sequences from a few lagoons were also found to be similar with other PSB, such as *Marichromatium* sp. (97%), *Thiolamprovum pedioforme* (93–100%), and *Thiobaca trueperi* (95–98%). *pufM* sequences amplified from enrichment and pure cultures were most similar to *T. roseopersicina* (93–96%). Carotenoid pigment concentrations, which were used as an indirect measure of purple bacteria levels in the wastewaters, were found to be positively correlated with salinity, nitrogen, total and volatile solids, and chemical oxygen demand; however, salinity could be the dominant factor influencing purple blooms. Due to the detection of PSB sequences in all lagoons, our findings suggest that the non-purple lagoons may have been purple in the past or may have the potential to become purple in the future.

Core Ideas

- PSB are often associated with reduced odors from livestock lagoons.
- PSB were found in both purple and non-purple dairy wastewaters.
- All dairy lagoons contained gene sequences similar to that of *Thiocapsa roseopersicina*.
- It may be possible to stimulate the growth of PSB in non-purple ponds without inoculation.

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PHOTOTROPHIC MICROORGANISMS, which reside in aquatic, benthic, and terrestrial environments, contain pigments that allow them to use light as an energy source. Anoxygenic photosynthesis among prokaryotes (in contrast to oxygenic photosynthesis) occurs in purple and green bacteria but does not result in the production of oxygen (Madigan and Martinko, 2006). Anoxyphototrophs, such as purple sulfur bacteria (PSB) and some purple nonsulfur bacteria (PNSB), use reduced sulfur compounds (e.g., hydrogen sulfide [H_2S], elemental S), thiosulfate, and molecular hydrogen as electron donors in photosynthesis (Dilling et al., 1995; Asao et al., 2007). Purple sulfur bacteria can also photoassimilate a number of simple organic compounds in the presence of sulfide, including volatile fatty acids, alcohols, and intermediates of the citric acid cycle (Rees et al., 2002; Zaar et al., 2003).

The occurrence of PSB in livestock waste management systems has been presented in the peer-reviewed literature for a few decades (Sletten and Singer, 1971; Wenke and Vogt, 1981; Goh et al., 2009). Lagoons that display a distinctive purple or pink color generally contain abundant growth of PSB, which have a distinctive growth advantage over PNSB in sulfide-rich environments (Chen et al., 2003). The coloring is a result of light-harvesting pigments (i.e., bacteriochlorophyll *a* or *b* and carotenoids) present in the bacteria (Madigan and Martinko, 2006). Organisms identified in livestock lagoons were *Thiocapsa roseopersicina* (McFarlane and Melcer, 1977), *Thiopedia rosea* (Wenke and Vogt, 1981; Freedman et al., 1983), and *Thiolamprovum pedioforme* (Goh et al., 2009). Compared with non-purple livestock lagoons, those that are purple are less likely to be considered an odor nuisance (Koelsch et al., 1997; Byler et al., 2004). This is related to the ability of purple bacteria to use H_2S and volatile organic compounds that are known odorants (Caumette, 1993; Guyoneaud et al., 1998).

The practical significance of purple lagoons is that the bacteria aid in the reclamation of the manure wastewaters. In addition to odor reduction, PSB have been documented to cause significant reductions of biological oxygen demand, ammonia, and phosphorus in swine waste (Earle et al., 1984). Because blooms only occur in some livestock waste management systems, attempts have been made to elucidate the conditions required for optimal

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Abbreviations: COD, chemical oxygen demand; EC, electrical conductivity; ORP, oxidation–reduction potential; PCR, polymerase chain reaction; PNSB, purple nonsulfur bacteria; PSB, purple sulfur bacteria.

growth of PSB (Wenke and Vogt, 1981; Chen et al., 2003; McGarvey et al., 2005). McGarvey and coworkers (2009) found that PSB growth in dairy lagoons could be induced by circulating the wastewater. To provide additional knowledge on this topic, the objectives of this study were to determine the occurrence of PSB in purple and non-purple dairy wastewaters and to identify possible linkages between the wastewater chemical and physical properties and purple blooms.

Materials and Methods

Wastewater Storage Lagoons

Seven wastewater lagoons at five open-lot dairies in south-central Idaho were selected for this study (Table 1). Samples were collected on a monthly basis from June to November, except from lagoon D2 (July–November), lagoon D3 (October and November), and lagoon E (June, September–November). Using a telescopic rod and a sterilized container, samples were collected just below the lagoon surface. Approximately eight 500-mL samples from the perimeter of each lagoon were composited in a sterile 4-L container. The containers were transferred to the laboratory in a cooler with ice packs and stored under refrigeration at 5°C and processed within 24 h of collection.

Analysis of the Wastewaters

The pH, electrical conductivity (EC), oxidation–reduction potential (ORP), and dissolved oxygen of field samples were determined using a 556 Multiprobe System (YSI Inc.). Total ammoniacal nitrogen, total solids, and volatile solids were determined according to Standard Methods 4500-NH_3, 2540 B, and 2540 E, respectively (Eaton et al., 2005). Total Kjeldahl nitrogen and chemical oxygen demand (COD) were performed using USEPA methods 351.2 and 410.4, respectively (USEPA, 1993).

Carotenoid pigments were extracted and quantified using the method of McGarvey et al. (2009). In brief, 1 mL of well-mixed wastewater was centrifuged at 14,000 *g* for 10 min. The supernatant was removed, and the pellet was suspended in 1 mL of methanol and incubated overnight at 5°C. After a centrifugation step as previously described, the methanol was discarded, and the pellet was washed with 0.1 mL of methanol. The methanol was once again discarded after centrifugation, and the pellet was suspended in ethyl acetate and allowed to extract overnight at 5°C. The sample was centrifuged, and the resulting extract was analyzed using a spectrophotometer. The carotenoid concentration was calculated by dividing the observed absorbance maximum (OD = 492 nm) of the extract by the molar extinction coefficient of spirilloxanthin (97 nmol L^{-1} cm^{-1}) at 492 nm.

Enrichment and Isolation of Purple Sulfur Bacteria

Purple sulfur bacteria in the dairy wastewaters were enriched to verify the presence of PSB in the dairy wastewaters (all months) and to produce biomass for whole-cell spectral scans and the extraction of DNA. Enrichment cultures were prepared by inoculating clear-glass, 40-mL serum bottles (Wheaton) containing Medium 1 (Pfennig and Trüper, 1981) with 100 μL of a well-mixed wastewater sample. In addition to sodium sulfide at 130 mg L^{-1}, the medium was supplemented with sodium thiosulfate at 670 mg L^{-1}. The serum bottles were capped with butyl rubber stoppers and aluminum seals. Inoculated bottles were incubated at about 30°C on a rotator mixer (10 rpm) under a 67 W incandescent light bulb (11 μmol s^{-1} m^{-2}) for 12 h d^{-1}.

To isolate pure colonies of PSB, the dairy wastewaters were prepared using the agar shake dilution method as described by Pfennig and Trüper (1981). After incubation for several weeks under incandescent light at 30°C, select colonies were removed and cultivated in Medium 1 as described above. The pure cultures were prepared for extraction of DNA as described below.

Extraction of DNA

The method of Dungan et al. (2012) was used to extract community DNA from all dairy wastewater samples. In brief, the wastewater samples were washed twice with cold phosphate-buffered solution and centrifuged at 10,000 *g* for 10 min between steps. The final pellet was transferred to a bead beating tube from a FastDNA SPIN Kit for feces (MP Biomedicals LLC) and processed using a FastPrep FP120 instrument at a speed setting of 6 m s^{-1} for 45 s. The DNA was eluted with 100 μL of tris(hydroxymethyl)aminomethane buffer (1%, w/w) and stored at −20°C until analyzed by polymerase chain reaction (PCR). DNA was extracted from enrichment and pure cultures (November samples) using the same conditions above, except the bacterial pellet was not washed with phosphate-buffered solution.

Polymerase Chain Reaction Amplification and Denaturing Gradient Gel Electrophoresis

Two PCR primer sets were used to target gene sequences within purple phototrophic bacteria. The primer pair Chr986f (5′-AGC CCT TGA CAT CCT CGG AA-3′) and GC1392r

Table 1. Details of the open-lot dairies, manure handling systems, and wastewater lagoons.

Dairy	Total cows	Cow breed	Manure handling system	Wastewater source	Lagoon ID	Lagoon stage†	Approximate lagoon surface area	Purple‡
							m^2	
1	5,000	Holstein	scrape	parlor	A	primary	10,320	no
2	1,500	Holstein	scrape	parlor	B	primary	2,749	yes
3	400	Holstein	scrape	parlor	C	primary	1,899	yes
4	3,300	Holstein, Jersey	scrape	parlor	D1	secondary	19,480	no
					D2	primary	2,472	no
				feed lane	D3	primary	869	yes
5	8,000	Holstein	flush	parlor, feed lanes	E	secondary	18,712	yes

† Secondary lagoons captured overflow from the primary lagoons.

‡ Lagoons were considered purple if they exhibited purple or pink coloring and had detectable levels of carotenoid pigment.

(5′-ACG GGC GGT GTG TAC-3′) was used to amplify a conservative 16S rRNA gene sequence within *Chromatiaceae*, the main family of PSB (Overmann et al., 1999). The primer pair pufM.557f (5′-CGC ACC TGG ACT GGA C-3′) and pufM.750r (5′-CCC ATG GTC CAG CGC CAG AA-3′) was used to amplify the *pufM* gene, which encodes a protein for the M subunit of the photosynthetic reaction center in purple sulfur and nonsulfur bacteria (Corson et al., 1999; Achenbach et al., 2001). A 40-nucleotide GC clamp was attached to the 5′ end of each reverse primer.

The PCR mixtures were prepared with 2 μL of DNA template, 0.3 μmol L^{-1} of each primer, 25 μL of AmpliTaq 360 Gold PCR Master Mix (Applied Biosystems), and molecular-grade water to a final volume of 50 μL. The conditions for Chr986f–GC1392r were 95°C for 10 min, then 40 cycles of 94°C for 30 s (15 cycles at 65°C for 1.5 min followed by 25 cycles at 58°C for 1 min) and 72°C for 40 s, with a final extension of 72°C for 7 min. The thermocycler conditions for pufM.557–pufM.750R were 95°C for 10 min, then 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The amplification products were visualized in 2% agarose gels with ethidium bromide staining.

Denaturing gradient gel electrophoresis was performed using the DCode Universal Mutation Detection System (Bio-Rad) to separate gene targets for sequencing. The PCR amplification products (45 μL) were loaded onto 8% (w/v) polyacrylamide gels, which were prepared with a linear gradient of 30 to 70% denaturant (100% denaturant is defined as 7 mol L^{-1} urea and 40% [v/v] formamide). The gels were electrophoresed in 1× Tris-acetate-EDTA buffer (Bio-Rad) for 3 h at 60°C and 180 V. After staining the gels with ethidium bromide, select bands were excised using an ethanol-cleaned scalpel. The bands were then placed into individual 0.65-mL sterile tubes with 20 μL of molecular-grade water. Ten microliters of the eluate was then used as template DNA for PCR using the above primers and thermocycler conditions, except that the GC clamp was omitted.

DNA Sequencing

The reamplified bands were directly sequenced in one direction using the Sanger method on an ABI 3730xl DNA Analyzer (Applied Biosystems). All sequencing was performed by TACGen. Identification of sequences was performed using the Basic Local Alignment Search Tool in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All unique 16S rRNA gene sequences have been deposited in GenBank under the accession numbers KP294456 through KP294496. The *pufM* gene sequences amplified from the wastewater community DNA, enrichments, and pure isolates have been provided as supplemental material because they were <200 bp and cannot be submitted to GenBank (see supplemental Dataset S1).

Statistical Analysis

To determine the relationship between the wastewater properties and carotenoid concentrations, Pearson correlation coefficients were calculated using the CORR procedure of SAS (SAS Institute, 2008). Statements of statistical significance were based on a $P < 0.05$.

Results and Discussion

Wastewaters from lagoons at open-lot dairies containing 400 to 8000 cows were investigated in this study (Table 1). All manure, including manure waste from the milking parlors, was diverted (after solids separation) to the lagoons, some of which were secondary lagoons to catch overflow. The chemical properties of the wastewaters are provided in Table 2. The pH of the wastewaters was above neutral at 7.5 to 8.6, whereas the EC was greatest (indicating a high salinity level) in the smaller lagoons (i.e., B, C, and D3) where wastewater inputs were low; thus, evaporation effects were evident. The mean ORP at less than −200 mV indicates that wastewaters were anoxic at the surface, although semioxic conditions (ORP > −100 mV) were recorded in lagoons A and D1 in June (data not shown). Most of the wastewater lagoons (except D1 and D2) also exhibited a purple color during the study (Fig. 1). The color of the wastewaters is a result of carotenoid pigments found in purple phototrophs; therefore, we quantified the pigments to track the growth pattern of purple bacteria. Carotenoid data presented in Table 3 indicate that purple bacteria levels were higher in certain lagoons (e.g., B, C, D3, and E) and that overall growth tended to be greatest during the summer, which coincides with increased solar radiation and water temperatures.

Based on the data presented in Table 2, it appears that certain wastewater chemical and physical properties might be enhancing the growth of PSB in the lagoons. To determine relationships between the carotenoid concentrations (i.e., an indirect measure of purple bacteria levels) and wastewater properties, Pearson correlation coefficients (r) were calculated. Carotenoid concentrations were found to be significantly and positively correlated

Table 2. Average chemical and physical properties of the dairy wastewater composite samples.

Lagoon ID	Months sampled	Physical properties†								
		pH	EC	ORP	DO	TAN	TKN	TS	VS	COD
			dS m^{-1}	mV	mg L^{-1}					
A	June–Nov.	8.0 (0.2)‡	2.9 (0.2)	−205 (92)	0.9 (0.9)	98 (21)	174 (14)	2,793 (230)	1,355 (243)	1,916 (171)
B	June–Nov.	8.5 (0.2)	9.8 (3.0)	−265 (84)	0.5 (0.3)	66 (31)	267 (25)	10,759 (3,297)	3,677 (772)	5,226 (1,178)
C	June–Nov.	8.4 (0.2)	10.1 (0.9)	−275 (87)	0.4 (0.2)	167 (85)	407 (38)	11,439 (1,226)	4,392 (570)	5,966 (604)
D1	June–Nov.	8.4 (0.4)	3.6 (0.4)	−227 (140)	2.3 (1.7)	30 (7)	94 (11)	3,236 (244)	1,099 (113)	1,108 (72)
D2	July–Nov.	7.9 (0.5)	3.0 (0.7)	−304 (118)	1.8 (2.0)	108 (50)	161 (40)	2,457 (550)	1,171 (233)	1,835 (526)
D3	Oct.–Nov.	8.6 (0.2)	15.9 (0.3)	−370 (79)	1.3 (0.8)	307 (160)	691 (83)	18,858 (1,637)	6,817 (354)	11,579 (218)
E	June, Sept.–Nov.	8.1 (0.3)	6.8 (0.7)	−272 (104)	1.1 (0.6)	287 (105)	461 (125)	7,038 (1,562)	3,532 (1,190)	5,246 (2,100)

† COD, chemical oxygen demand; DO, dissolved oxygen; EC, electrical conductivity; ORP, oxidation–reduction potential; TAN, total ammoniacal nitrogen; TKN, total Kjeldahl nitrogen; TS, total solids; VS, volatile solids.

‡ Values in the parentheses are SD.

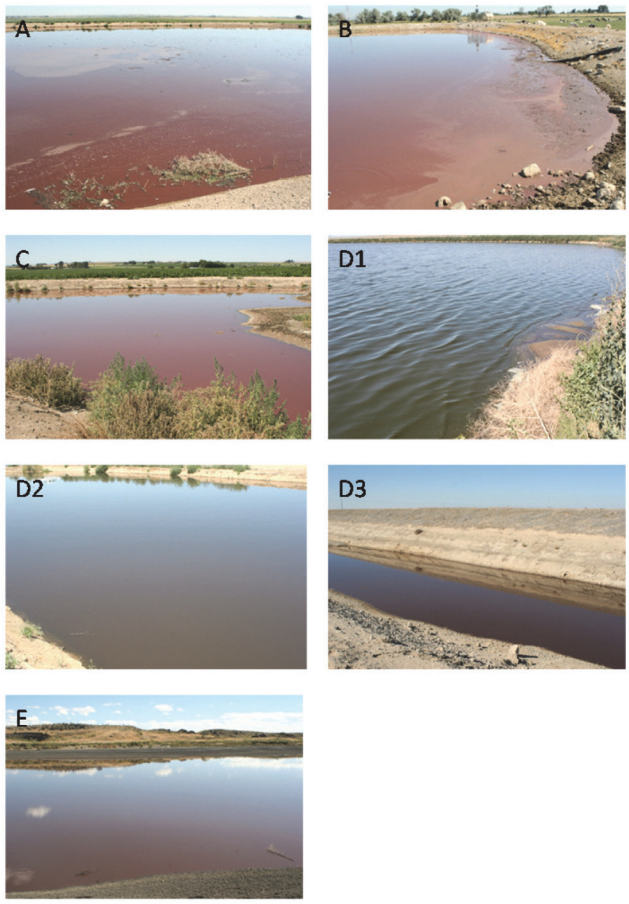

Fig. 1. Purple and non-purple dairy wastewater lagoons investigated in this study. Select photographs from various months are presented to highlight the purple coloring. The lagoon IDs are in the upper left hand corner of each image.

with EC ($r = 0.78$; $P < 0.0001$), TKN ($r = 0.40$; $P < 0.01$), TS ($r = 0.80$; $P < 0.0001$), volatile solids ($r = 0.66$; $P < 0.0001$), and COD ($r = 0.61$; $P < 0.0001$). These results should be interpreted cautiously, however, because the purple bacteria biomass is potentially contributing to the elevated nitrogen, solids, and COD concentrations but not salinity. For confirmation, it would be necessary to compare the properties of the wastewater entering the lagoon with that which is already present. Because the inlet pipes to the storage lagoons were below the surface in most cases, we were unable to make this comparison.

In a study of swine manure lagoons, purple lagoons had lower numerical concentrations of salinity, alkalinity, ammonium, and COD when compared with non-purple lagoons, but the differences were not statistically significant (Chen et al., 2003). As noted by these authors and by our personal observations, manure wastewaters may never turn purple or may fail to turn purple on a regular basis. Gilley et al. (2000) suggested that limited growth of purple bacteria in some anaerobic swine lagoons could be related to dietary copper, which results in the production of potentially inhibitory levels of sulfide. Because copper sulfate footbath waste is often discarded in dairy wastewater lagoons (Ippolito et al., 2010), the impacts of copper on purple blooms deserves further attention. We did not acquire information from the dairy workers regarding the disposal of footbath waste in the lagoons investigated in this study.

Wastewater samples were inoculated into media specific for the phototrophic cultivation of PSB using sulfide. Despite there being no purple color in some lagoons, the enrichments from these lagoons displayed distinctive purple or pink coloring, which is a positive indication of PSB growth (Fig. 2). The enrichment for lagoon D1 also contained green algae and was slightly brown as a result, but purple bacteria were visible in the serum bottles when the cells were allowed to settle. Regardless of purple status and month during which the samples were collected, PSB could be enriched from all wastewaters (data not shown). This additional information indicates that phototrophic microbes, such as PSB, were present in the dairy wastewaters even when they were not undergoing a bloom. However, even with abundant solar radiation in this high desert region, it is unclear why some lagoons did not turn purple despite our ability to enrich purple bacteria. Evidence from this study and other published reports suggests that it is likely related to the wastewater properties.

Ultraviolet-visible absorption spectra of whole cells from the enrichments indicate the presence of bacteriochlorophyll *a* and carotenoids pigments (Fig. 3). Absorption maxima at 375, 800, 856, and 897 nm are characteristic of bacteriochlorophyll *a*, whereas maxima at 486, 516, and 554 nm are typical of carotenoids of the spirilloxanthin series. Very similar results were obtained from a spectroscopic analysis of whole cells belonging to *Thiocapsa* species, including *T. roseopersicina* (Asao et al., 2007; Núñez-Cordona et al., 2008). The spectrum of enrichment D1 also displays a peak at 680 nm, which indicates the presence of chlorophyll *a* from green algae, which dominated growth in lagoon D1.

To tentatively identify PSB in the dairy wastewaters, gene sequences were compared with database entries in GenBank (Table 4). Using 16S rDNA primers specific to *Chromatiaceae*, sequences from gel bands affiliated with all seven lagoons were

Table 3. Concentration of carotenoid pigments in the dairy wastewaters.

Month	Lagoon ID						
	A	B	C	D1	D2	D3	E
	mg L^{-1}						
June	0.41†	2.34	2.42	0.38	–‡	–	0.11
July	0.55	4.37	5.44	0.24	0.03	–	–
Aug.	0.88	6.22	7.56	0.07	0.15	–	–
Sept.	0.61	9.95	7.22	0.09	0.07	–	0.84
Oct.	0.69	1.56	6.96	0.11	0.05	4.53	1.45
Nov.	0.23	2.32	1.22	0.04	0.03	2.79	0.21

† Concentrations reported as mg spirilloxanthin L^{-1} of wastewater. Average of three analytical replicates.

‡ No wastewater samples were collected during that month.

Fig. 2. Enrichment cultures about 2 wk after being spiked with the dairy wastewaters. Images from the November sampling campaign, with lagoon ID below each serum bottle.

92 to 100% similar to *Thiocapsa* sp., with one band sequence from lagoon A being 92% similar to *T. roseopersicina*. Sequences from lagoon D2 were also similar with the PSB *Thiobaca trueperi* (95–98%) and *Marichromatium* sp. (97%). *Thiobaca trueperi* has been isolated from sediment in a freshwater lake (Rees et al., 2002), and various species of *Marichromatium* have been isolated from soil, river sediment, and marine water (Arunasri et al., 2005; Kumar et al., 2007; Sucharita et al., 2010).

Sequences from lagoons B, C, D1, and D3, based on the *pufM* gene primer set, were determined to be 93 to 100% similar to *T. pedioforme* (Table 4). Gene sequences from *T. pedioforme* have been identified in purple dairy wastewater lagoons along with that of another PSB, *Rhabdochromatium marinum* (McGarvey et al., 2005). In a study of three swine waste lagoons, analysis of cellular morphology and 16S rRNA gene sequences indicated that all lagoons were dominated by *T. pedioforme* (Goh et al., 2009). In our study, sequences from six of seven lagoons were also 91 to 97% similar to *T. roseopersicina*, which has been identified in a number of anoxic lagoon systems, such as those that handle swine waste and meat packing, animal skin processing, and municipal wastewaters (Freedman et al., 1983). Because the *pufM* primer set targets both purple sulfur and nonsulfur bacteria, our results suggest that PSB may be the predominant purple bacteria in the lagoons because none of the sequences matched with PNSB. Although some of sequences were similar to uncultured bacteria (Table 4), it is possible that these could be from PNSB. However, all *pufM* gene sequences from enrichment

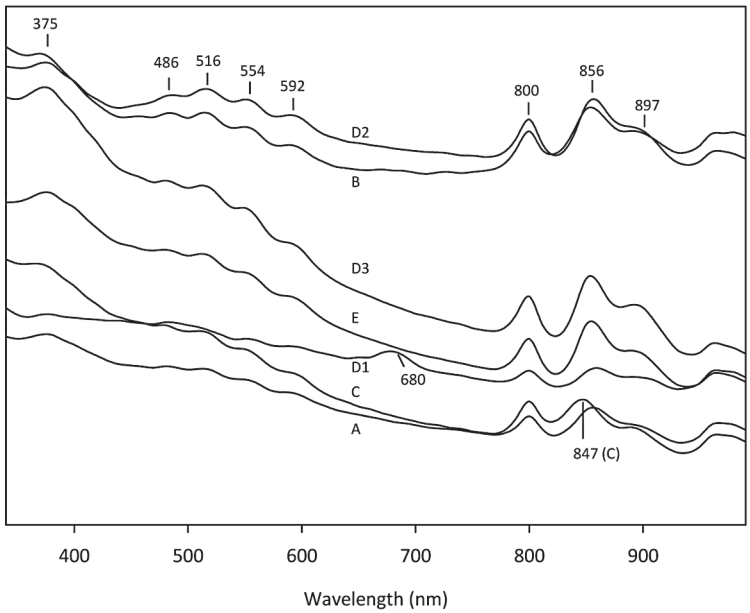

Fig. 3. Ultraviolet-visible absorption spectra (340–990 nm) for whole cells from the November enrichment cultures. The numbers denote the position of the peaks and shoulders. Each spectrum is also labeled with the lagoon ID from which the enrichment was generated.

Table 4. GenBank matches for amplified gene sequences from the dairy wastewaters.

Primer pair	Lagoon ID	Closest GenBank match	Accession no.	No. band sequences	Similarity
					%
Chr986f–GC1392r	D2	*Marichromatium* sp.	FN552743	1	97
	D2	*Thiobaca trueperi*	AJ404007	2	95–98
	A	*Thiocapsa roseopersicina*	Y12364	1	92
	A, D1, D2, D3	*Thiocapsa* sp.	FN293073	7	92–99
	A, B, C, D1, D2, E	*Thiocapsa* sp.	FN293074	12	95–100
	A, B, C, D1, E	*Thiocapsa* sp.	FN293078	10	94–99
	A, B, C, D1, D2, D3	*Thiocapsa* sp.	KC702856	9	97–99
pufM.557r–pufM.750r	B, C, D1, D3	*Thiolamprovum pedioforme*	FN257152	9	93–100
	B, C, D1, D2, D3, E	*T. roseopersicina*	AJ544223	17	93–97
	A, E	uncultured bacterium	AB486021	5	97–99
	D1, D2	uncultured bacterium	AB510466	2	90–91
	A, D1, E	uncultured proteobacterium	GU080280	7	90–94
	D1	uncultured bacterium	HG003770	1	91
	D1	uncultured bacterium	HQ222684	1	90

and pure cultures had the highest similarity (91–96%) with *T. roseopersicina* (supplemental Dataset S1), providing additional evidence that members of the genus *Thiocapsa* or a closely related bacterium are common among these dairy wastewaters.

In conclusion, genes from PSB were detected in all of the dairy wastewater lagoons despite the fact that a few did not display an obvious purple or pink color. In many cases, the 16S rRNA and *pufM* gene sequences were <100% similar to GenBank sequences, indicating that some of the wastewater PSB could represent unknown genera or species. Regardless, our findings suggest that the non-purple lagoons may have been purple in the past or may have the potential to become purple in the future. If one excludes the potential influence of purple bacteria biomass on nitrogen, solids, and COD levels in the wastewaters, high salinity could be influencing the blooms because the purple lagoons had the highest average EC values. Because of the known odor reduction benefits associated with purple bacteria blooms in livestock waste storage systems, additional research should be undertaken to determine if physical and/or chemical manipulation can be used to enhance the growth of PSB in non-purple dairy wastewaters.

Supplemental Material

Dataset S1. *pufM* gene sequences from the wastewaters (WW), enrichments (Enr), and isolates (Isol).

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